THE EFFECT OF LABELING INTENSITY, ESTIMATED BY REAL-TIME CONFOCAL LASER SCANNING MICROSCOPY, ON FLOW CYTOMETRIC APPEARANCE AND IDENTIFICATION OF IMMUNOCHEMICALLY LABELED MARINE DINOFLAGELLATES

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THE EFFECT OF LABELING INTENSITY, ESTIMATED BY REAL-TIME CONFOCAL LASER SCANNING MICROSCOPY, ON FLOW CYTOMETRIC APPEARANCE AND IDENTIFICATION OF IMMUNOCHEMICALLY LABELED MARINE DINOFLAGELLATES[1]

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ABSTRACT

Two different fluorescein isothiocyanate (FITC) conjugates were used to analyze the effect of labeling intensity on the flow cytometric appearance of marine dinoflagellates labeled with antibodies that specifically recognized the outer cell wall. Location of the labeling was revealed by epifluorescence and real-time confocal laser scanning microscopy using an anti-rabbit IgG/FITC-conjugated secondary antiserum. Flow cytometric measurements showed that cells of Prorocentrum *species labeled this way could not always be distinguished from unlabeled cells. The labeling intensity increased several times when a biotinylated anti-rabbit IgG secondary antiserum was used in combination with a streptavidin/FITC conjugate. Flow cytometry indicated that the labeling intensity had increased 50%, which resulted in an improved separation of clusters of labeled and unlabeled cells.*

Key index words: biotin–streptavidin system; confocal laser scanning microscopy (CLSM); flow cytometry; labeling intensity; Pyrrophyta; toxic marine phytoplankton

During the last two decades, an increase in the occurrence of nuisance and toxic algal blooms has been reported worldwide (Anderson 1989). Some argue that the incidence of such blooms may increase further due to continuing eutrophication and other pollution and general global changes (Smayda 1990). Along the western European coasts, including the Dutch part of the North Sea, toxic dinoflagellates such as *Dinophysis, Gymnodinium, Gyrodinium,* and *Alexandrium* have frequently been reported lately (Tangen 1979, Kat 1985, Partensky and Sournia 1986, Fraga 1988, Larsen and Moestrup 1989, Peperzak 1990, Reid et al. 1990). Of the diatoms, species of *Nitzschia* have always been reported in the North Sea (Horwood et al. 1982, Leewis 1985, Reid et al. 1987). However, since 1991, *Nitzschia pungens* Grunow forma *pungens* Hasle has been identified (R. Koeman, pers. commun.). This organism is closely related to two domoic acid-producing species, *N. pungens* Grunow f. *multiseries* Hasle, which has caused deaths of humans in eastern Canada (Todd 1990), and *Pseudonitzschia australis* Frenguelli, which killed sea birds in California (Fritz et al. 1992, Work et al. 1992). A representative of another class, the prymnesiophyte *Chrysochromulina polylepis* Manton et Parke, caused mass fish kills and death of benthic fauna in Scandinavian coastal waters in 1988 (Rosenberg et al. 1988, Dahl et al. 1989).

Because of the severe effects of natural biotoxins on invertebrates, fish, and other constituents of the food chain, several methods have been developed to identify toxic algae and to detect their toxins. These methods include bioassays (Kat 1987), high-performance liquid chromatography (Lee et al. 1987, Oshima et al. 1989) and gas chromatography techniques (Hallegraeff et al. 1991), ELISA (Cembella and Lamoureux 1992), and immunochemical staining methods (Anderson and Cheng 1988, Sako et

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al. 1992, Vrieling et al. 1992). Because most of these techniques are rather time-consuming, our goal has been to develop a method that combines immunochemical recognition of target cells (Campbell et al. 1983, Shapiro et al. 1989, Anderson et al. 1990, van Bleijswijk et al. 1991) with fast-flow cytometric counting of labeled cells (Yentsch 1981, Phinney and Cucci 1989). This method may allow the detection of toxic marine phytoplankton before a bloom has started, thus providing an early warning system. We showed earlier that labeled cells of naked dinoflagellates can be distinguished effectively from unlabeled ones (Vrieling et al. 1992). In organisms that display a high degree of chlorophyll autofluorescence, the intensity of the green fluorescein isothiocyanate (FITC) fluorescence obtained from immunolabeling is not sufficient to overcome the high background (Vrieling et al. 1992). We have now improved the labeling intensity, defined as the amount of immunochemically bound FITC, by using a biotin–streptavidin system. We examined the effect of this procedure on the resolution of our detection system by real-time confocal laser scanning microscopy (CLSM) and flow cytometry using several toxic and nontoxic dinoflagellate cultures.

MATERIAL AND METHODS

Strains of *Gyrodinium* cf. *aureolum* Hulbert (strain Iroise) and *Gymnodinium nagasakiense* Takayama & Adachi (strain Buzen '85-2) were obtained from F. Partensky, Roscoff, France. Both strains and isolates of *Prorocentrum micans* Ehrenberg (a strain originally obtained from M. Elbraechter, List/Sylt, Germany), *Prorocentrum minimum* Pavillard (strain W30min1), and *Gymnodinium simplex* (Lohman) Kofoid et Swezy (strain CCMP418 from the Provasoli/Guillard Culture Collection, West Boothbay Harbor, Maine) were maintained in f/2-Si (Guillard and Ryther 1962) at temperatures of 16° C and a 14:10 h LD cycle with an irradiance of approximately 75 $\mu E \cdot m^{-2} \cdot s^{-1}$.

Antisera. Three antisera were used. Two antisera were directed against purified cell walls (Pm1) and extruded trichocystal cores (Pm2) of *P. micans*, respectively. Whole paraformaldehyde-killed cells of *G. simplex* at a concentration of 10^5 cells $\cdot mL^{-1}$ were used as antigen for the third antiserum (GSIM). Prior to injection, the antigens were emulsified in Freund's adjuvant. Immunization of chinchilla rabbits was achieved by subcutaneous injections at intervals of 10 days. Test bleeds obtained after 30 days of immunization were analyzed for antibody activity using an immunofluorescence assay, as described previously (Campbell et al. 1983, Campbell 1988). A final boost injection was necessary to obtain a stronger anti-cell wall serum (Pm1). Specificity of the three antisera was tested by cross-reactivity experiments with a series of available phytoplankton species. Antiserum Pm1 was genus-specific for *Prorocentrum* and was used for staining of *P. micans* and *P. minimum* (Vrieling et al. 1992). Antiserum Pm2 (specific for trichocyst-bearing dinoflagellates) was used for staining of *G.* cf. *aureolum* and *G. nagasakiense* because of the strong affinity to outer cell wall components of these species (Vrieling et al. 1992). Antiserum GSIM showed some minor cross-reactivity with other phytoplankton species (unpubl. results). The working dilution of 1:100 of both primary and secondary antisera revealed suitable fluorescence intensities, while false positives did not occur as determined by immunofluorescence dilution series (unpubl. results). Preimmune sera of all three antisera showed no significant labeling of algal cells.

Immunochemical staining. Phytoplankton cells were fixed *in situ* as described previously (Vrieling et al. 1992). Cells in 200-mL cultures were either fixed with 2% (w/v) paraformaldehyde or 2% (v/v) glutaraldehyde final concentration for 1.5 h at room temperature. After fixation, cells were harvested by mild centrifugation (1000–2000 rpm), washed twice with phosphate-buffered saline (PBS) containing 1% (w/v) bovine serum albumin, carefully resuspended in 500 μL PBS supplemented with primary antisera at a 1:100 dilution, and incubated for 1.5 h at 37° C. After incubation, the samples were washed three times with PBS, resuspended in 500 μL of this same buffer containing a 1:100 dilution of secondary antiserum goat anti-rabbit IgG/FITC (GAR/FITC) or donkey anti-rabbit IgG/biotin (DAR/biotin), and incubated for 1.5 h at 37° C. Using the biotinylated secondary antiserum, additional wash steps with PBS were required before and after incubation of streptavidin/FITC (1 h at a 1:100 dilution in PBS at 37° C). Control experiments to reveal nonspecific labeling were performed 1) in the absence of primary antisera, with only secondary antiserum (GAR/FITC) or streptavidin/FITC, and 2) in the absence of secondary antiserum after binding of the primary one to examine nonspecific binding of streptavidin/FITC as the fluorescent conjugate. All samples were examined with a Zeiss Axioskop epifluorescence microscope equipped with a blue excitation bandpass filter (BP 450–490 nm) and an emission cut-off at 520 nm.

Confocal laser scanning microscopy. Quantitative CLSM was performed with a prototype of the ODYSSEY® (Noran Instr., Wisconsin) as a video-rate confocal laser scanning microscope (Draaijer and Houpt 1988, Houpt and Draaijer 1989). The excitation wavelength of a 40-mW Argon-ion laser was 488 nm, while the power of the laser, measured above the objective lens, was adjusted to 0.22 mW. The emission filter was either a longpass LP580 for chlorophyll or a bandpass BP520–560 for FITC. To quantify labeling intensity, the gray values in the images caused by immunochemical staining were compared to gray values obtained from images of solutions with a known amount of FITC. These solutions were from a calibration line, prepared by diluting FITC in buffer at pH 8. The amount of fluorescence was determined as gray values measured by photon counting (as a function of FITC concentration) for a photomultiplier voltage of 500 V. The validity of this method was confirmed by comparison of the lifetime images of the solutions and the algae (Buurman et al. 1992). For three cells per algal strain (*P. micans*, *G.* cf. *aureolum*, and *G. nagasakiense*) the highest gray values of 10 randomly chosen spots per focal plane were averaged and corrected for the photomultiplier voltage of the calibration line and the background emission.

Flow cytometry. Flow cytometric measurements were performed with the Optical Plankton Analyser (OPA), a flow cytometer specially designed for phytoplankton analysis (Dubelaar et al. 1989, Peeters et al. 1989). A Coherent Innova 90-5 Argon-ion laser, operating at 488 nm for excitation of FITC, was used. Green fluorescence was measured by separating emitted light with the following filter setup: reflection on a 495-nm shortpass dichroic mirror, filtering by two longpass filters (an LP500 nm and an LP520 nm), reflection on a 550-nm longpass dichroic mirror, and filtering with a 544-nm shortpass dichroic filter. To minimize scattered laser light, a sheet polarizer (Melles-Griot 03FPG007) was used perpendicular to the polarization of the laser. Orange fluorescence was separated by filtering with a 550-nm longpass filter, reflection on a 650-nm longpass dichroic mirror, and filtering with a 634-nm shortpass dichroic filter. Red fluorescence, separated by filtering with a 670-nm longpass filter, was used as the triggering parameter. The resulting OPA parameters and filter setup used for the experiments are explained in Table 1 and Figure 1, respectively. For positive identification of labeled cells, green FITC fluorescence was compared to the red autofluorescence of chlorophyll. Appearance in bivariate plots and mean fluorescence of green and orange were determined for different species with three different labeling conditions: 1) unlabeled, 2)

TABLE 1. *Parameters of the OPA. FB = fluorescence measured at blue excitation.*

Parameter	$I_{excitation}$ (nm)	$I_{emission}$ (nm)	Related to
FBG (Green)	488	520–544	FITC
FBO (orange)	488	550–634	Phycoerythrin
FBR (red)	488	650–750	Chlorophyll
TOF (time of flight)	488	650–750	Particle length

labeled with GAR/FITC, and 3) labeled by the biotin–streptavidin system. Fluorescence spectra of *P. minimum* and *G. nagasakiense* under the preceding conditions were obtained from a Spex Fluorolog 2 fluorescence spectrophotometer as described by Hofstraat et al. (1992).

Materials. GAR serum conjugated with fluorescein-isothiocyanate (GAR/FITC) was obtained from Sigma. Biotinylated species-specific DAR (DAR/biotin) serum and streptavidin/FITC were obtained from Amersham.

RESULTS

Confocal laser scanning microscopy. Epifluorescence microscopy and CLSM showed that labeling of the algae with different antisera was confined to the outer cell wall of fixed intact cells. For *P. minimum*, *G.* cf. *aureolum*, and *G. nagasakiense* (Fig. 2A–C), FITC fluorescence was randomly distributed around the outside of the cells, whereas pores in the cell wall (Fig. 2D) and the site near the anterior spine of *P. micans* (Fig. 2E) were labeled. No significant nonspecific labeling of the outer cell wall of cells fixed with paraformaldehyde was observed in controls, whereas detectable nonspecific labeling was noticed in some glutaraldehyde-fixed cells (not shown).

Examination of images obtained from labeled *P. micans* with antiserum Pm1 and *G. nagasakiense* cells with Pm2 revealed that, at photomultiplier (PMT) voltages of 680 and 660 V, a sufficient signal was obtained for FITC fluorescence when anti-rabbit IgG/FITC was applied (Fig. 3A, C). However, leaking chlorophyll autofluorescence interfered, as can be seen by gray features (arrows in Fig. 3A, C),

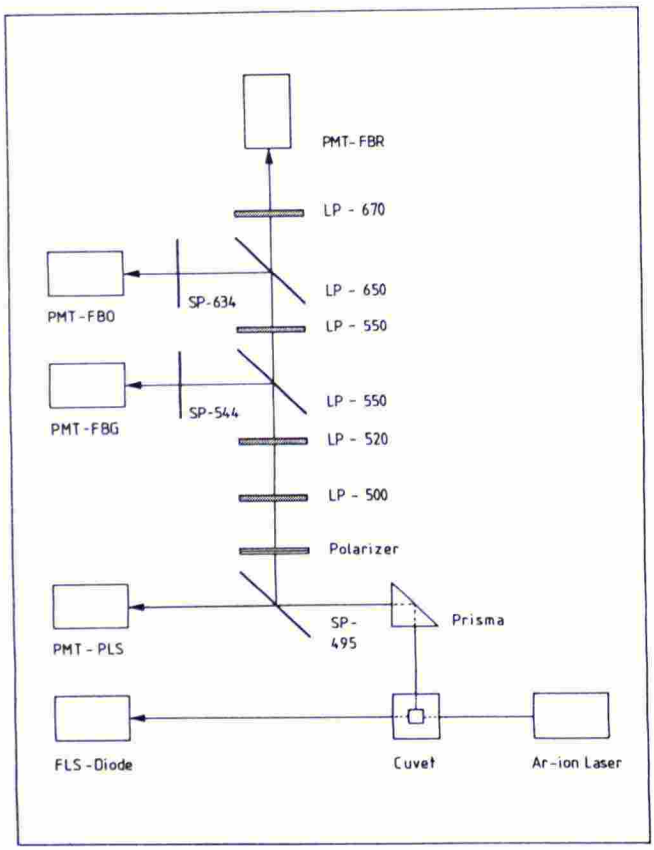

FIG. 1. Schematic presentation of the filter setup for flow cytometric detection of algal cells immunochemically labeled with FITC.

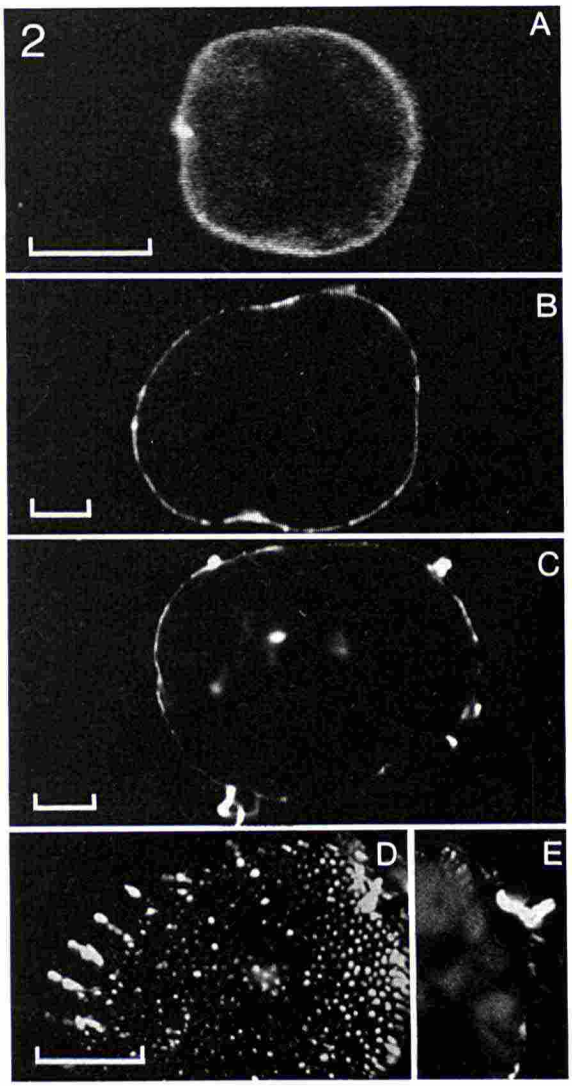

FIG. 2. Micrographs of labeled dinoflagellates obtained by CLSM using the biotin–streptavidin system. A) *P. minimum*, B) *G. aureolum*, C) *G. nagasakiense*, and D) *P. micans*. E) Part of the cell shown in D scanned at a lower focus plane. *Prorocentrum* species are labeled with Pm1; the others are labeled with Pm2. Horizontal field of view is 80 μm, NA = 1.25. Scale bars = 10 μm.

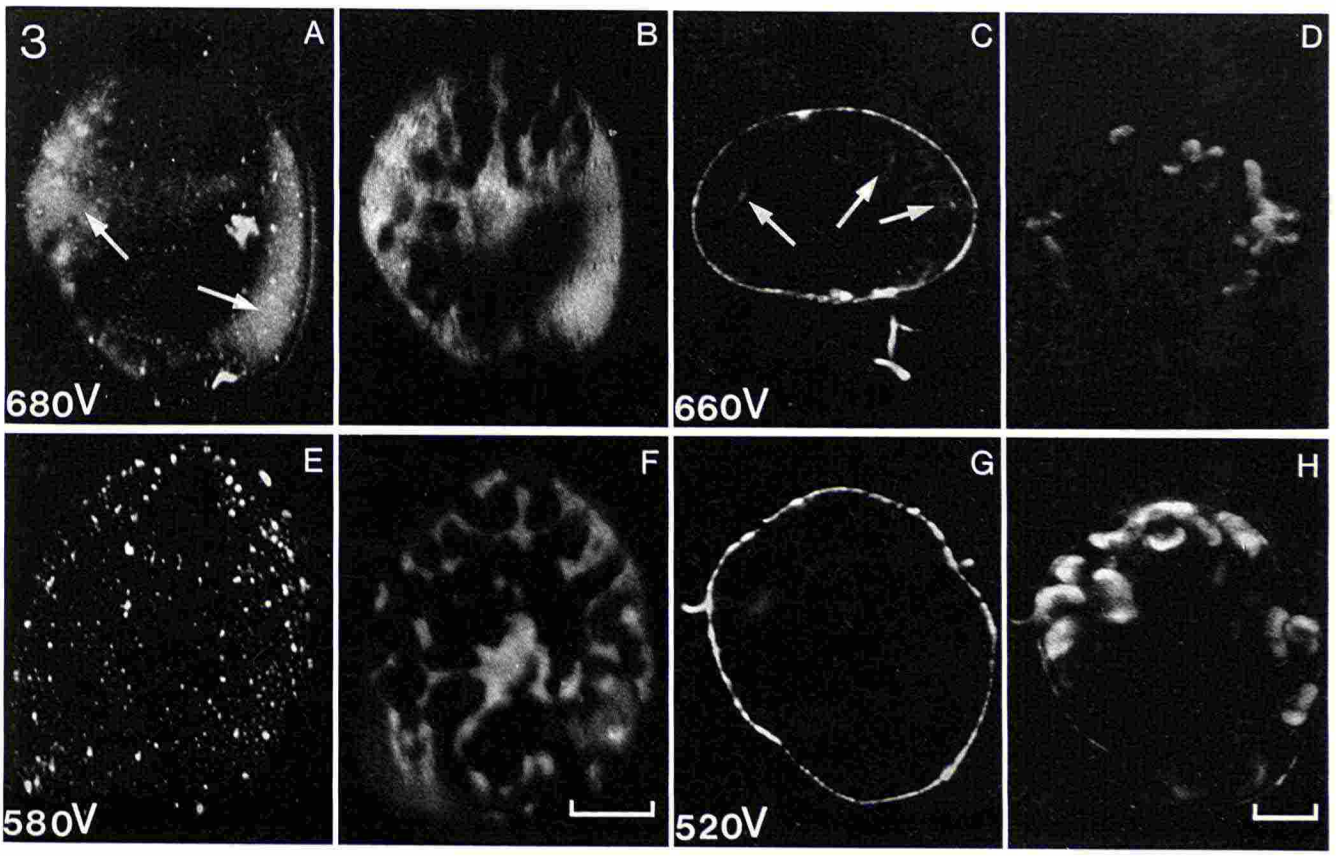

FIG. 3. Micrographs of *P. micans* (A, B, E, F: labeled with Pm1) and *G. nagasakiense* (C, D, G, H: labeled with Pm2) showing the effect of labeling intensity on PMT sensitivity. FITC fluorescence in cells treated with A, C) GAR/FITC and E, G) the biotin–streptavidin system. B, D, F, H) Chlorophyll autofluorescence of the same focal plane. Note that at lower PMT voltages no interfering chlorophyll autofluorescence occurred in E and G compared to A and C (arrows) at higher voltages. Horizontal field of view is 80 μm, NA = 1.25. Scale bars = 10 μm.

coinciding with chloroplast images that were obtained when chlorophyll autofluorescence was examined in the same focal plane (Fig. 3B, D). When the biotin–streptavidin system was applied, the PMT voltages for reaching optimal images decreased to 580 and 520 V (Fig. 3E, G). In this case, leaking chlorophyll fluorescence was not noticed at the same spots as the chloroplast images (Fig. 3F, H). Comparable results were obtained for *G.* cf. *aureolum* (not shown).

Estimates of labeling intensity show the difference of FITC fluorescence of cells labeled with GAR/FITC and the biotin–streptavidin system (Table 2).

TABLE 2. *CSLM; mean fluorescence intensity (μM FITC) of triplets determined from the FITC calibration line, for randomly chosen (n = 10) high intensive stained spots per focal plane.*

Species	GAR/FITC	DAR/biotin streptavidin/FITC	Enhancement factor (F)
Prorocentrum micans	1.6 ± 0.8	4.8 ± 2.3	3.0
Gyrodinium cf. *aureolum*	2.4 ± 0.8	10.0 ± 3.0	4.2
Gymnodinium nagasakiense	2.3 ± 0.6	14.0 ± 3.0	6.9

Mean values of 10 spots per focal plane per cell were corrected for differences in PMT voltages and background and represent the amount of FITC (in μM) determined from the FITC calibration line (Fig. 4). Enhancement factors (F) differed: *P. micans* dis-

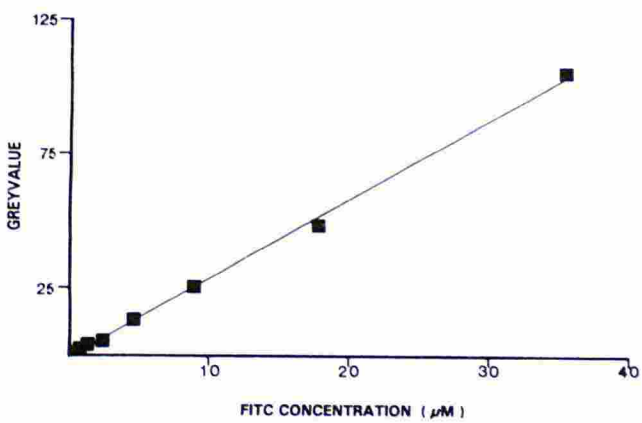

FIG. 4. Gray value, estimated by CLSM, as a function of FITC concentration (μM), recorded for FITC dilutions measured at a PMT voltage of 500 V.

TABLE 3. *Mean linear fluorescence intensity values as measured by flow cytometry of duplicates (n = 5000) for FBG; 520–544 nm.*

	Unlabeled	GAR/FITC	DAR/biotin streptavidin/FITC
P. micans	14,950 ± 9966	28,948 ± 25,832	27,720 ± 31,189
P. minimum	1847 ± 1126	12,948 ± 15,041	18,792 ± 29,635
G. simplex	6791 ± 3590	29,990 ± 22,078	34,709 ± 41,814
G. cf. *aureolum*	14,456 ± 8056	37,512 ± 19,578	73,470 ± 34,327
G. nagasakiense	1977 ± 844	11,273 ± 5801	21,608 ± 15,368

played the lowest with F = 3, followed by *G.* cf. *aureolum* with F = 4.2 and *G. nagasakiense* with F = 6.8.

Flow cytometry. Mean fluorescence of green (FBG) and orange (FBO) was determined for duplicate measurements of 5000 events for each species tested at different labeling conditions (Tables 3, 4). The labeled naked dinoflagellates *G. nagasakiense*, *G.* cf. *aureolum*, and *G. simplex* showed very high degrees of green fluorescence, which made them readily distinguishable from unlabeled cells by their separated clusters in flow cytometric bivariates. For example, clusters of unlabeled (light blue) and GAR/FITC-labeled (dark blue) cells of *G. nagasakiense* were well separated in the bivariate plots of FBG and red fluorescence (FBR) (Fig. 5A). The biotin–streptavidin system (pink cluster in Fig. 5A) improved the distinction of unlabeled and labeled cells due to a higher level of green fluorescence compared to cells labeled with GAR/FITC (Fig. 5A). Histograms of the number of events as a function of FBG gave three well-separated intensity peaks, albeit with an overlap for peaks 2 and 3 (Fig. 5C). For *P. minimum* and especially *P. micans*, discrimination between unlabeled and GAR/FITC-labeled cells was hardly possible, whereas clusters were sufficiently separated using the biotin–streptavidin system (Fig. 5B). Histograms show three peaks, of which peaks 2 and 3 were very close, resulting in an almost complete overlap (Fig. 5D). Unexpected was the increase of FBO, comparable to FITC, for labeled cells (Table 4).

Determination of the fluorescence spectra of unlabeled and labeled cells of *P. minimum* (Fig. 6A) and *G. nagasakiense* (Fig. 6B) showed a considerable amount of fluorescence (but not a clearcut maximum) in the region of 550–634 nm, which explains the occurrence of the high FBO noticed in flow cytometric measurements. Note that unlabeled cells showed no orange autofluorescence at all. The increase of relative fluorescence (Fig. 7) was determined from the areas of FITC fluorescence (520–544 nm) and FBO (550–634 nm) beneath the spectral curves of Figure 5. The enhancement of FITC fluorescence when different conjugates were applied is evident. FBO showed only a minor increase, while in flow cytometric measurements (shown in Table 4) very high intensity values were noticed.

DISCUSSION

In this attempt to enhance immunofluorescent staining of algal cells, the difference between the "normal" procedure (fluorochrome-conjugated secondary antisera) and the biotin–streptavidin system, revealed by epifluorescence microscopy, is evident. However, localization of the label on phytoplankton may still be difficult. CLSM allowed a clear distinction between FITC fluorescence and interfering chlorophyll autofluorescence so that the label associated exclusively with the cell wall could be easily distinguished. However, when the amount of fluorescent probe applied is insufficient, the chlorophyll fluorescence in phytoplankton cells will hinder the localization of antigens and estimation of fluorescence intensities, due to poor antisera or lack of exposed outer cell wall antigens.

For flow cytometry and CLSM, we used samples that were fixed with freshly prepared paraformaldehyde, because glutaraldehyde showed distinct disadvantages. First, it significantly weakened chlorophyll autofluorescence, so flow cytometric triggering of autotrophic phytoplankton is affected; preservation according to Vaulot et al. (1989) should prevent this. Second, glutaraldehyde induces an increase of nonspecific labeling (Shapiro et al. 1989, Anderson et al. 1990).

We clearly show that compared to biotin–streptavidin FITC images in "normal" FITC images, the contribution of chlorophyll autofluorescence is much higher, due to differences in FITC concentration.

TABLE 4. *Mean linear fluorescence intensity values as measured by flow cytometry of duplicate measurements (n = 5000) for FBO; 550–634 nm.*

	Unlabeled	GAR/FITC	DAR/biotin streptavidin/FITC
P. micans	9906 ± 5325	38,703 ± 48,720	42,182 ± 64,826
P. minimum	4822 ± 2662	43,477 ± 50,351	68,289 ± 101,848
G. simplex	4717 ± 666	20,103 ± 13,955	32,996 ± 39,790
G. cf. *aureolum*	3840 ± 1540	26,205 ± 14,972	35,710 ± 22,167
G. nagasakiense	7636 ± 3662	37,417 ± 19,026	77,165 ± 50,896

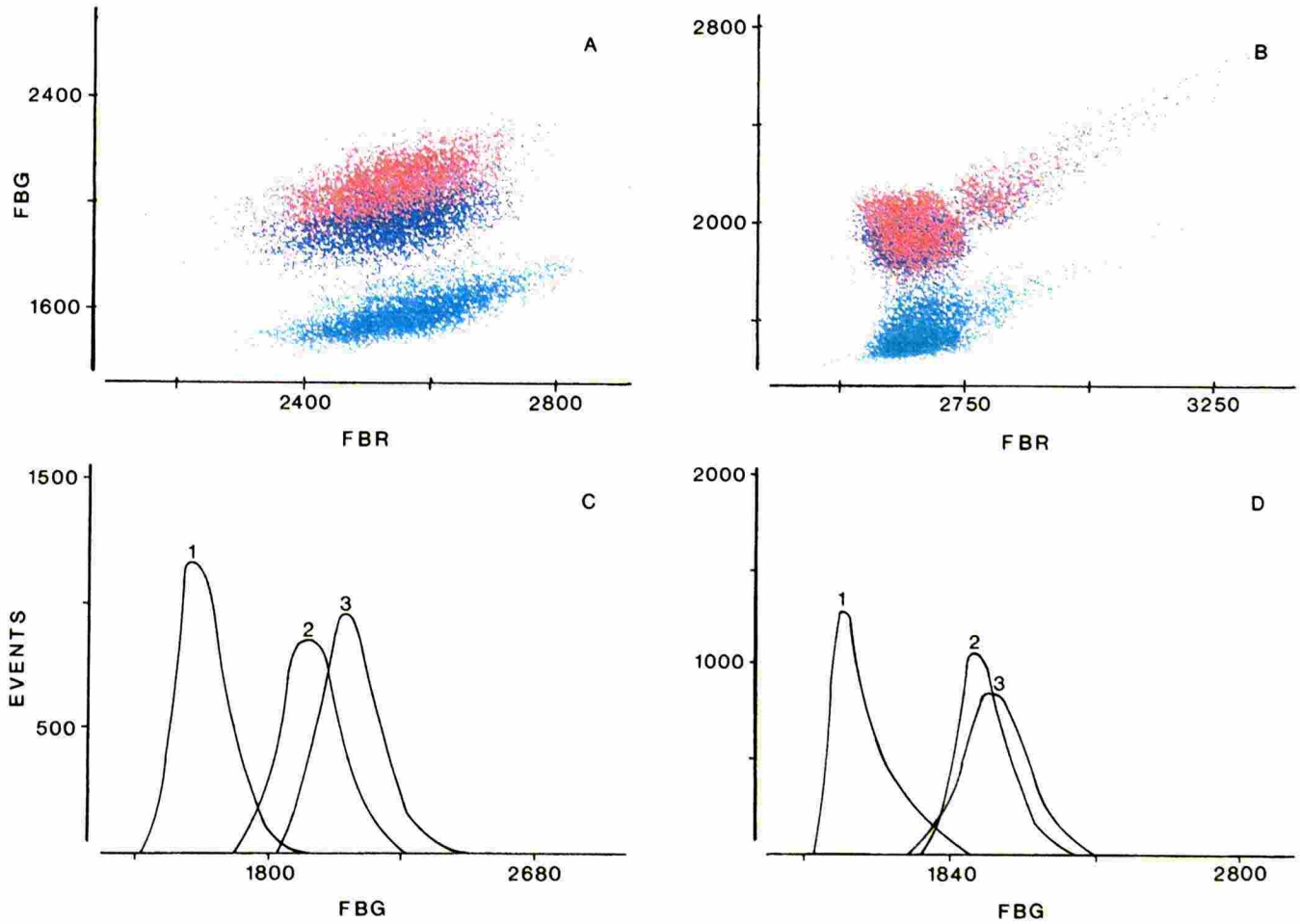

FIG. 5. Flow cytometric data of duplicate measurements on unlabeled and labeled cells of *G. nagasakiense* (A, C: labeled with Pm2) and *P. minimum* (B, D: labeled with Pm1). A, B) Appearance of cells in the bivariate plots of green (FBG) and red (FBR) fluorescence (both log-scaled). C, D) Histograms of the number of events versus mean FBG fluorescence (log-scaled) of duplicate measurements (n = 5000) showing three detectable peaks. Labeling conditions are represented by A, B) a light blue, dark blue, and pink cluster or C, D) as peaks 1, 2, and 3 for unlabeled cells, cells labeled with GAR/FITC, and cells labeled with the biotin–streptavidin system, respectively.

Samples with a low FITC concentration need high PMT voltages to obtain suitable images, while crosstalk between FITC and chlorophyll channels becomes more visible. This chlorophyll autofluorescence as well as background laser light may interfere with FITC measurements during flow cytometry.

By using the biotin–streptavidin system, thus increasing the amount of dye coupled per bound antibody, the sensitivity of our method improved because the ratio of FITC to chlorophyll autofluorescence increased. In case of both conjugates, apart from an enhanced FBG from FITC, FBO increased as well. Fluorescence spectra did not show a clearcut maximum around 590 nm for either *un*labeled or labeled cells, so occurrence of orange autofluorescence could be excluded. By comparing filter sets, we learned that the FITC bandpass filter used for the flow cytometer had a range of 520–544 nm, whereas for CLSM a wider range (520–560 nm) was used. From the fluorescence spectra (Fig. 6), it is clear that the FITC maximum is at 520 nm, followed by a long tail ending at about 600 nm. Thus, when measuring FBO in a range from 550 to 634 nm, part of the FITC fluorescence is detected in the orange channel and becomes masked for the green channel. Most probably, maximal resolution of clusters has not been reached; adjustment of the filter settings (i.e. by measuring FBG from 520 up to 560 nm) may lead to even better results.

The standard deviations of the data presented in Tables 3 and 4 are high. Explanations of this are 1) variability in cell size of the species used, related to life-cycle phenomena (Blackburn et al. 1989, Partensky and Vaulot 1989, Partensky et al. 1991), 2) variability in intensities of FITC fluorescence of individual cells (e.g. due to improper ratios of antisera/amount of cells), 3) clustering of cells during the labeling procedure or measurements (this was, however, never noticed when samples were checked by epifluorescence microscopy), and 4) limitation of the threshold of the flow cytometer, especially at high cell concentrations, so two or more cells can

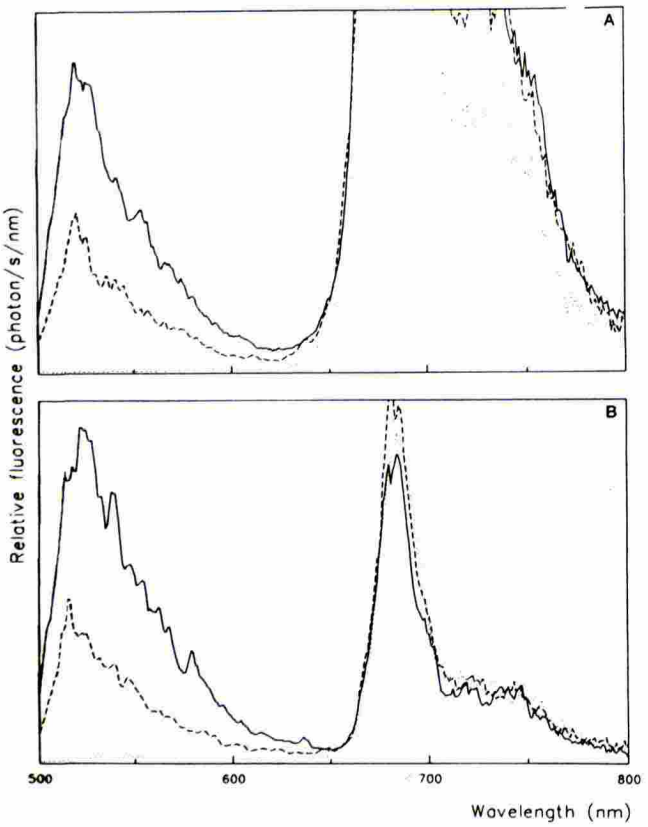

FIG. 6. Fluorescence spectra at an excitation wavelength of 488 nm of unlabeled and labeled cells of A) *P. minimum* and B) *G. nagasakiense*; (·····) = unlabeled cells, (— — —) = GAR/FITC-labeled cells, and (——) = cells labeled with the biotin–streptavidin system. For each species, cell densities were approximately equal for the labeling conditions.

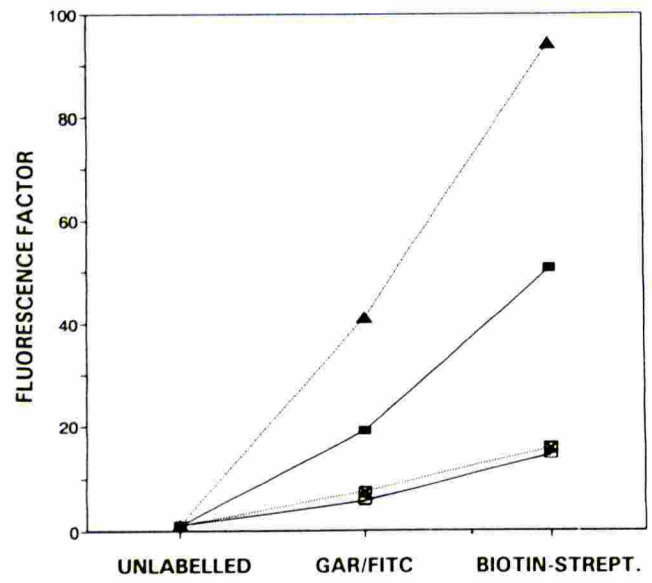

FIG. 7. Fluorescence factor, determined from the fluorescence spectra of *P. minimum* and *G. nagasakiense* at the labeling conditions for limited filter areas (520–544 nm for FBG and 550–634 nm for FBO); (▲) = FBG and (⊠) = FBO for *G. nagasakiense*, (■) = FBG and (□) = FBO for *P. minimum*.

follow each other at a very close distance in the sample cuvet resulting in identification of only one particle with a higher fluorescence value.

The variations in increase of labeling intensity with the biotin–streptavidin system, expressed as enhancement factor F, between species (see Table 2 and Fig. 7) was expected due to the experimental setup because no effort was taken to prepare each species under equal conditions. Indeed, our principal aim was simply to improve the resolution of our detection method. Therefore, it is not surprising that variations in local binding of the primary antisera and availability of the amount of binding sites for the secondary antibodies induced variations in the enhancement factor; cell concentrations and antiserum dilutions were not equal for each sample. The low enhancement factor observed for *P. micans* most probably is due to disturbed optimal binding of streptavidin/FITC at the pores in the cell wall, which was prevented by steric hindress. With enhanced labeling, this may easily occur because we used a biotinylated secondary antiserum with biotin molecules coupled to the IgG by spacer arms, so complexes of multivalent streptavidin/FITC and the biotinylated heavy chains of our secondary IgGs may have formed.

In CLSM experiments, the concentration of FITC was determined in one focal plane per cell, whereas for flow cytometry, the fluorescence intensity of labeled cells was determined for individual cells (assuming that the threshold was sufficient). With flow cytometry, however, only part of the cell is illuminated by the focused, oval, laser spot (1 mm in width and 5 μm in height) during the time of flight (Dubelaar et al. 1989, Peeters et al. 1989), so complete excitation of FITC from labeled cells cannot be expected. During measurements, CLSM recovered more FITC fluorescence than the flow cytometer because of the wider wavelength range (16 nm wider for CLSM), as shown in the fluorescence spectra. All these facts may explain the difference in enhancement factor of the two techniques: approximately 1.5 for flow cytometry and ranging from 3.0 to 6.9 for CLSM.

Nevertheless, despite its lower labeling intensity, flow cytometric detection of algae labeled according to the "normal" immunochemical procedure resulted in very clear discrimination of labeled and unlabeled cells of the naked dinoflagellates. For *Prorocentrum* species, however, distinction was only sufficient when labeling intensity was enhanced by applying the biotin–streptavidin system. This sensitive method will be the basis of our ongoing efforts to set up a rapid and unambiguous identification method for toxic marine phytoplankton at early bloom stages.

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